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Alcohol and Cocaine Exposure Modulates ABCB1 and ABCG2 Transporters in Male Alcohol-Preferring Rats

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Abstract

Two efflux transporters, ATP-binding cassettes B1 (ABCB1) and G2 (ABCG2), are highly expressed in the endothelial cells of the brain, where they regulate the bioavailability and distribution of several endogenous and xenobiotic compounds. However, whether ABCB1 or ABCG2 has any link with drug dependence, drug withdrawal effects, or the incidence of adverse effects in drug abuser is not known. In this study, we determined the effects of voluntary ethanol consumption following repeated exposure to cocaine or vehicle on the relative mRNA and protein expression of *Abcg2*/ABCG2 and *Abcb1*/ABCB1 in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) of male alcohol-preferring (P) rats. Male P rats were allowed free choice access to ethanol (15 and 30% *v*/*v*) and water for 5 weeks to establish baseline drinking behavior. The following week, rats were either injected with 20 mg/kg i.p. of cocaine or saline, once a day, for 7 days. The relative mRNA and protein expression of *Abcb1*/ABCB1 and *Abcg2*/ABCG2 in the NAc and mPFC were significantly decreased in ethanol-saline- and ethanol-cocaine-exposed rats compared to control rats that received neither ethanol nor cocaine. Thus, prolonged exposure to commonly abused drugs, ethanol and cocaine, alters the expression of *Abcb1*/ABCB1 and *Abcg2*/ABCG2 mRNA and protein levels in brain areas that play a role in drug dependence.

Keywords Ethanol · Cocaine · ABCB1 · ABCG2 · NAc · mPFC

Introduction

Interaction between drugs (e.g., drug-drug interactions; DDI), including drugs of abuse, can contribute to severe adverse effects. Some DDI may involve alterations in the expression or function of ATP-binding cassette (ABC) proteins [1]. These DDI may result from the non-linear elimination characteristics of the ABC transporters, as well as the competition between the substrates of ABC transporters. Based on amino acid sequence homology, the ABC transporter family has been categorized into seven main subtypes (ABCA–ABCG) and these transporters are involved in either the efflux or influx of endogenous and exogenous compounds [2]. ABC transporters, such as ABCG2 (breast cancer resistance protein, BCRP), ABCB1 (P-glycoprotein, P-gp), and ABCC2 (multidrug resistance proteins 2, MRP-2) [3, 4], are expressed in the canalicular membrane of liver cells and the apical membranes of the epithelial barriers in the kidney and intestine. The overexpression of these efflux transporters is correlated with the reduction in the systemic concentrations of their substrates [3, 5]. In addition, the overexpression of ABCB1 and ABCG2 in the brain capillary endothelial cells of the blood brain barrier (BBB) limits the penetration of certain drugs into the brain, thereby limiting or abrogating their therapeutic efficacy [6, 7].

Drug addicts commonly receive pharmacological treatments for various other medical conditions, such as certain antiretroviral and antipsychotic drugs, that are ABCB1 and ABCG2 substrates [1, 8]. These treatments could consequently alter responses to drugs of abuse, or conversely a history of illicit drug use might alter responses to these treatment medications, resulting in deleterious drug-drug interactions or even contributing to the addictive process. There is also, of course, substantial co-abuse of multiple illicit substances, particularly the co-use of ethanol with most other drugs of abuse. Previous

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studies, using in vitro and in vivo models, have identified roles of specific ABC proteins in transporting various drugs of abuse [9–11]. For example, it has been shown that buprenorphine is transported across the BBB via an ABCB1-mediated efflux transport system [12]. Cocaine was also found to be a substrate for ABCB1 transporters in bovine brain endothelial cells [10]. Moreover, methadone inhibits the activity of ABCB1 in vitro in Caco-2 cells [13] and in rodents [14, 15], which might therefore affect subsequent responses to methadone and other ABCB1 substrates.

Such effects on the expression of ABC transporters in response to substrate exposure or other molecular signals would be expected to be most apparent after chronic treatments. It has been reported that short-term (4 days) exposure to ethanol decreases the cellular expression of ABCG2 in cortical progenitor cells [16]. Disulfiram, a drug approved for the treatment of alcohol abuse, interacts with ABCB1 binding sites, which may induce drug resistance [17]. Thus, concurrent exposure to ethanol and disulfiram could produce adverse effects due to interactions at the level of ABC transporters. Unlike other drugs of abuse, ethanol would not be expected to be a substrate for ABC transporters and to instead cross the BBB by passive diffusion [18], but may affect the function of these transporters in other ways. For instance, chronic ethanol exposure affects the level of transcriptional factors and neuroinflammatory biomarkers that may regulate the expression of ABC transporters [19–21].

Cocaine is a psychostimulant drug that produces its addictive effects via alteration of neuronal function/activity in brain areas hypothesized to mediate reward and reinforcement [22]. Cocaine crosses the BBB by passive diffusion to some extent, but more importantly, by the proton antiporter flux system [23, 24]. However, data suggest that cocaine may also be transported by ABCB1 [10]. In addition to the pharmacodynamic mechanisms that have been postulated to mediate the development of cocaine dependence [25, 26], it has been suggested that chronic exposure to cocaine affects brain proteins that may play a role in the pathogenesis and/or pathophysiology of other diseases [27, 28]. Indeed, cocaine affects chemokine receptors of the immune system [29], and these changes could be involved in the activation or repression of ABC transporters [30, 31]. Since long-term exposure to chemotherapeutic drugs increases the expression of ABC transporters in the brain, it might be thought that exposure to these drugs might influence subsequent responses to drugs of abuse that are substrates for these transporters. Similarly, a prior history of drug abuse might also affect subsequent responses to chemotherapeutic drugs. It is therefore important to investigate the effects of chronic exposure to drugs of abuse, including cocaine, on the expression of these transporters. To begin to address this question, the present study determined the direct effect of concurrent, repeated i.p. injections of cocaine and voluntary oral ethanol consumption on mRNA and protein expression of *Abcb1*/ABCB1and *Abcg2*/ABCG2 in the NAc and mPFC of male alcohol-preferring (P) rats. This dual regimen was chosen because of previous research demonstrating substantial changes in brain mechanisms influencing the neurotoxic effects of drugs of abuse [32, 33].

Materials and Methods

Subjects

Fifteen male alcohol-preferring (P) rats (21–30 days of age) were obtained from Indiana University, School of Medicine (Indianapolis, IN, USA) and were housed in the Department of Laboratory Animal Resources, University of Toledo, Health Science Campus. The animal protocol for this study was approved by the Institutional Animal Care and Use Committee of The University of Toledo and was in accordance with all National Institutes of Health guidelines for animal research, including the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, 1996).

Animals were grouped and housed in a vivarium room kept on a 12-/12-h light/dark cycle and maintained under controlled temperature (23 ± 2 °C) and humidity (50 ± 5%). The number of animals in each cage was chosen depending on the animals' weight in accordance with IACUC guidelines.

Behavioral Drinking Paradigm

The time line of voluntary ethanol consumption and repeated cocaine administration (20 mg/kg, i.p.) is illustrated in Fig. 1. At 75 days of age, rats were housed individually under controlled temperature (23 ± 2 °C) and humidity (50 ± 5%) conditions and were maintained on a 12-/12-h light/dark cycle. Rats had ad libitum access to food and water throughout the study. Ethanol-treated rats (groups 2 and 3 below; see Fig. 1) were exposed to a free choice ethanol drinking procedure (15 and 30% ethanol *v*/*v* and water, concurrently) for a period of 5 weeks. During the fourth week of ethanol consumption, ethanol and water intake were measured three times a week for 2 weeks. Rats consuming less than 4 g/kg/day of ethanol were excluded (three rats) from the study, as described in previous studies [32, 34]. On the following week, the ethanol-exposed P rats were randomly divided into two groups, so that there were three groups overall: (A) group 1 (water control group), exposed to water throughout the study and injected i.p. with 0.9% saline (vehicle); (B) group 2 (ethanol-saline group), exposed to ethanol and water throughout the study and injected i.p. with saline once daily for 7 days; and (C) group 3 (ethanol-cocaine group), exposed to ethanol and water throughout the study and injected with 20 mg/kg i.p. of cocaine once daily for 7 days.

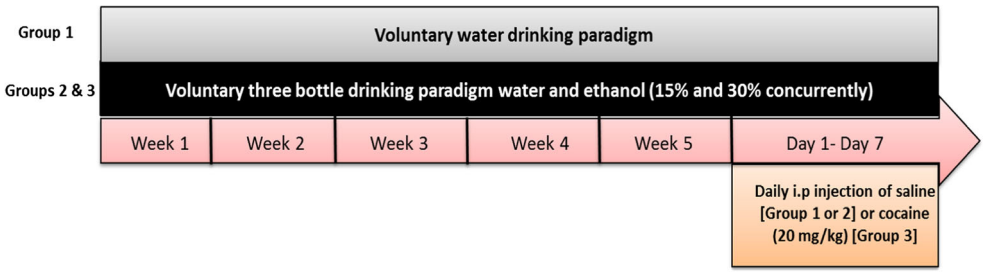

Fig. 1 The time line for voluntary ethanol drinking and repeated cocaine exposure

Brain Tissue Harvesting

Carbon dioxide (27%) was used to euthanize rats 24 h after the last cocaine or saline injection (and at the same time for control subjects). Rats were decapitated using a guillotine and the brains were removed, placed on dry ice until frozen and stored at − 80 °C until samples were collected. A cryostat apparatus, set at − 20 °C, was used for dissection of samples from the mPFC and NAc. Tissue sections were taken until the brain regions of interest could be identified using the Rat Brain Atlas [35]. The isolated brain regions (left and right sides) were stored at − 80 °C for immunoblot and quantitative polymerase chain reaction (PCR) analysis.

Western Blot

A lysis buffer (1 M Tris HCL, 3 M NaCl, 0.5 M EDTA, 10% NP-40, 10% Triton, 10% SDS) containing protease inhibitors (Thermo Scientific, Rockford, IL, USA) was used to lyse the brain tissues in preparation for Western blot analysis, as previously described [36]. A protein quantification assay was performed using a DC (detergent compatible) protein assay (Bio-Rad Laboratories, USA). Subsequently, equal amounts of extracted proteins from each sample were separated on 10% polyacrylamide gels. The proteins were transferred from the gels to PVDF membranes (Bio-Rad, Hercules, CA, USA). Subsequently, TBST (50 mM Tris HCl; 150 mM NaCl, pH 7.4; 0.1% Tween 20) containing 5% non-fat dry milk was used to block the membranes at room temperature for 30 min. The membranes were then exposed to one of the following primary antibodies: mouse anti-MDR1/ABCB1 (1:200, Novus Biological), or mouse anti-ABCG2/CD338 (1: 2000, Novus Biological) at 4 °C overnight. A loading control protein was assessed throughout the study using mouse anti-GAPDH (1: 5000; Cell Signaling Technology). On the second day, the membranes were washed five times with TBST. Subsequently, TBST in 3% non-fat dry milk was used to further block the membranes for 30 min. At room temperature, membranes were exposed to the secondary antibody, anti-mouse ABCB1, ABCG2, and GABDH (1:5000; Cell Signaling Technology) for 90 min. The membranes were washed five times with TBST, dried, and incubated with the developing kits to detect proteins (SuperSignal West Pico Chemiluminescent substrate, Rockford, IL, USA). The membranes were exposed to film (Kodak BioMax MR Film, Fisher Inc., Holiston, MI, USA) and the film was developed using an SRX-101A machine (Konica Minolta Medical and Graphic Inc.). To quantify the intensity of the detected bands, an MCID system (Imaging Research Inc., Ontario, Canada) was used and obtained values were expressed as a percentage of the relative ratio of the proteins of interest to GAPDH (100% water control value) as described previously [25, 37].

The gels in the Western blot analyses were run in triplicate and not all samples could be run on the same gel. Furthermore, there are many factors that can influence, by increasing or decreasing, the intensity of the blot in each run, including room temperature, the amount of protein loaded, the ratio of the developing kit, and the film exposure time. Hence, we standardized the expression of the protein of interest for each sample to the corresponding water control sample that was run in the same gel, under the same conditions, as described in several previous studies [32, 38–41] .

Real-Time, Quantitative PCR (RT-PCR, qPCR)

Triazol Reagent (Life Technologies, Carlsbad, CA, USA) was used to isolate total RNAs from the NAc and mPFC. Subsequently, using a verso cDNA synthesis kit (Thermo Scientific, Lithuania), reverse transcription (RT) was done according to the manufacturer's protocol. An iCycler (Bio-Rad laboratories, München, Germany) was used to perform Real-Time PCR (RT-PCR). RT-PCR was done using a reaction mixture of SYBR Green as a fluorescent dye (Bio-Rad Laboratories), a 1/20 volume of cDNA preparation as a template, and the appropriate primers for the genes of interest as shown in Table 1. A threshold cycle number (C_T) for each sample was obtained from the iCycler and was used to compare the relative amount of target mRNA in experimental groups with those of controls, using the $2^{-\Delta\Delta CT}$ method [33, 44]. Each sample was run in triplicate. In order to get ΔC_T, the mean C_T value for the control gene, GAPDH was subtracted from the mean C_T value of the gene of interest. The ΔC_T values for the control group (ethanol-naive) were then averaged and were subtracted from ΔC_T for the experimental groups to obtain $\Delta\Delta C_T$. The relative fold change from control was then expressed by calculating $2^{-\Delta\Delta CT}$ for each sample

Table 1 Primer sequence for rat *Abcb1*, *Abcg2*, and *Gapdh*

Gene	Primer	Sequence[a, b]
Abcb1	Forward primer	5′- GTGTTTCTAGATGGCAAAGA -3′
	Reverse primer	5′- CCACTCTGGTGTTGTATTTC -3′
2Abcg2	Forward primer	5′- AAGACCATGAAGCAAACAAG -3′
	Reverse primer	5′- ACACTGGTTGTTAGTCAGGA -3′
Gapdh	Forward primer	5′- CCCCCAATGTATCCGTTGTG -3′
	Reverse primer	5′- TAGCCCAGGATGCCCTTTAGT -3′

[a] *Abcb1* and *Abcg2* primer sequences were taken from [42]

[b] *Gapdh* primer sequences were taken from [43]

and the results were reported as the group mean fold change ± SEM.

Statistical Analyses

The relative mRNA and protein expression of *Abcb1*/ABCB1 to *Gapdh*/GAPDH and *Abcg2*/ABCG2 to Gapdh/GAPDH for water-control, ethanol-saline, and ethanol-cocaine groups were analyzed using a one-way ANOVA. Post hoc comparisons were made using the Newman-Keuls multiple comparison test. A priori significance level was set to $p < 0.05$.

Results

The Effect of Voluntary Ethanol Consumption and the Repeated Cocaine Administration on the Relative mRNA Expression of Abcb1 and Abcg2 in the NAc and the mPFC

The Relative mRNA Expression of Abcb1 and Abcg2 in the NAc

***Abcb1* mRNA** Statistical analysis indicated a significant decrease in the relative *Abcb1* mRNA expression [F (2, 12) = 5.454, $p = 0.0207$] in the NAc of rats that voluntarily consumed ethanol and in rats treated chronically with 20 mg/kg i.p. of cocaine after ethanol consumption, compared to ethanol-naïve P rats (Fig. 2 and Table 2, left). There was no significant difference between ethanol-treated groups resulting from cocaine exposure.

***Abcg2* mRNA** Statistical analysis revealed a significant decrease in the relative *Abcg2* mRNA expression [F (2, 12) = 7.549, $p = 0.0075$] in the NAc of rats that voluntarily consumed ethanol and in rats treated chronically with 20 mg/kg i.p. cocaine after ethanol consumption, compared to ethanol-naïve P rats (Fig. 2 and Table 2, right). There was no significant difference between ethanol-treated groups resulting from cocaine exposure.

The Relative mRNA Expression of *Abcb1* and *Abcg2* in the mPFC

***Abcb1* mRNA** A significant decrease in relative *Abcb1* mRNA expression [F (2, 12) = 8.254, $p = 0.0056$] occurred in the mPFC of rats that voluntarily consumed ethanol and in rats chronically treated with 20 mg/kg i.p. cocaine after ethanol consumption, compared to ethanol-naïve P rats in mPFC (Fig. 3 and Table 3, left). There was no difference between ethanol-treated groups resulting from cocaine treatment.

***Abcg2* mRNA** A significant decrease in relative *Abcg2* mRNA expression [F (2, 12) = 4.857, $p = 0.0285$] occurred in the mPFC of rats that voluntarily consumed ethanol and in rats chronically treated with 20 mg/kg i.p. of cocaine after ethanol consumption (Fig. 3 and Table 3, right). There was no difference between ethanol-treated groups resulting from cocaine treatment.

The Effect of Voluntary Ethanol Consumption and the Repeated Cocaine Administration on ABCB1 and ABCG2 Protein Expression in the NAc and the mPFC

ABCB1 and ABCG2 Expression in the NAc

ABCB1 Statistical analysis indicated that a significant decrease [F (2, 12) = 9.259, $p = 0.0037$] in relative ABCB1 expression occurred in the NAc of rats that voluntarily consumed ethanol and in rats treated chronically with 20 mg/kg i.p. cocaine after ethanol consumption, compared to ethanol-naïve P rats (Fig. 4 and Table 4, left). There was no difference between ethanol-treated groups resulting from cocaine treatment.

ABCG2 Statistical analysis indicated that a significant decrease [F (2, 12) = 20.078, $p < 0.0001$] in relative ABCG2 expression occurred in the NAc of rats that voluntarily consumed ethanol and in rats treated chronically with 20 mg/kg i.p. of cocaine after ethanol consumption, compared to ethanol-naïve P rats (Fig. 4 and Table 4, right). There was no difference between ethanol-treated groups resulting from cocaine treatment.

ABCB1 and ABCG2 Expression in the mPFC

ABCB1 Statistical analysis indicated that a significant decrease [F (2, 12) = 9.653, $p = 0.0032$] in relative ABCB1 expression occurred in the mPFC of rats that voluntarily consumed ethanol and in rats treated chronically with 20 mg/kg i.p. of cocaine after ethanol consumption, compared to ethanol-naïve P rats (Fig. 5 and Table 5, left). There was no difference between ethanol-treated groups resulting from cocaine treatment.

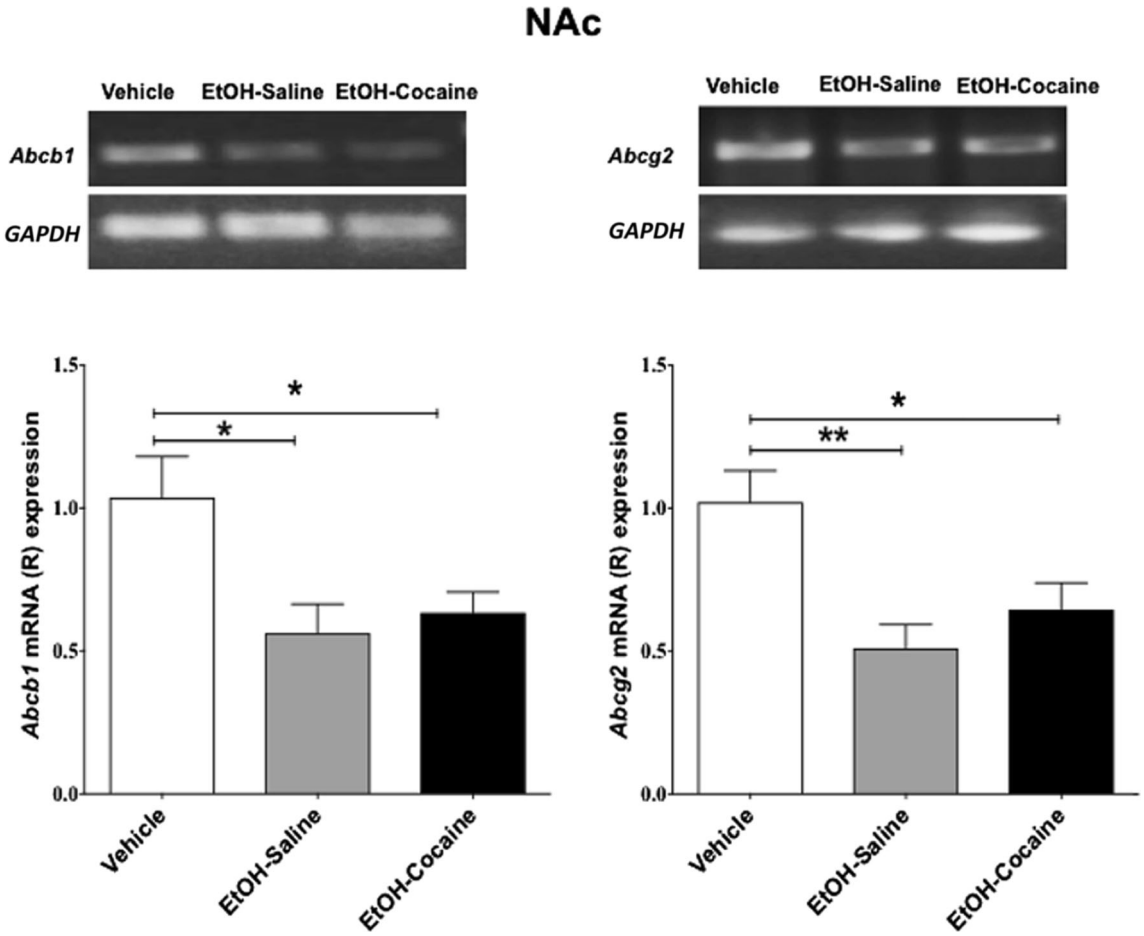

Fig. 2 Relative mRNA expression of *Abcb1* and *Abcg2* in the NAc following voluntary ethanol consumption and repeated cocaine exposure (mean ± SEM). **a** One-way ANOVA followed by the Newman-Keuls multiple comparisons test revealed a significant decrease in relative mRNA expression of *Abcb1* in the NAc. **b** One-way ANOVA followed by the Newman-Keuls multiple comparisons test revealed a significant decrease in relative mRNA expression of *Abcg2* in the NAc. (*$p < 0.05$, **$p < 0.01$), ($n = 5$ for each group)

ABCG2 Statistical analysis indicated that a significant decrease [$F(2, 12) = 19.567$, $p = 0.0002$] in relative ABCG2 expression occurred in the mPFC of rats that voluntarily consumed ethanol and in rats treated chronically with 20 mg/kg i.p. cocaine after ethanol consumption, compared to ethanol-naïve P rats (Fig. 5 and Table 5, right). There was no difference between ethanol-treated groups resulting from cocaine treatment.

Discussion

In this study, the voluntary consumption of ethanol, as well as concurrent ethanol and cocaine treatment, significantly decreased relative mRNA and protein expression of the ABCB1 and ABCG2 transporters in the NAc and mPFC of male P rats. To our knowledge, our study is the first to report that ethanol

Table 2 Relative mRNA expression of *Abcb1* and *Abcg2* in the NAc following voluntary ethanol consumption and repeated cocaine exposure expressed as mean ± SD.

Abcb1 mRNA expression				Abcg2 mRNA expression			
Vehicle		EtOH-saline	EtOH-cocaine	Vehicle		EtOH-saline	EtOH-cocaine
0.93		0.47	0.77	0.85		0.61	0.44
1.17		0.51	0.73	0.95		0.46	0.82
0.80		0.45	0.60	0.84		0.74	0.72
1.53		0.96	0.70	1.02		0.49	0.84
0.76		0.42	0.37	1.44		0.24	0.40
Mean ± SD	1.04 ± 0.32	0.56 ± 0.22	0.63 ± 0.16	Mean ± SD	1.02 ± 0.25	0.51 ± 0.19	0.64 ± 0.21

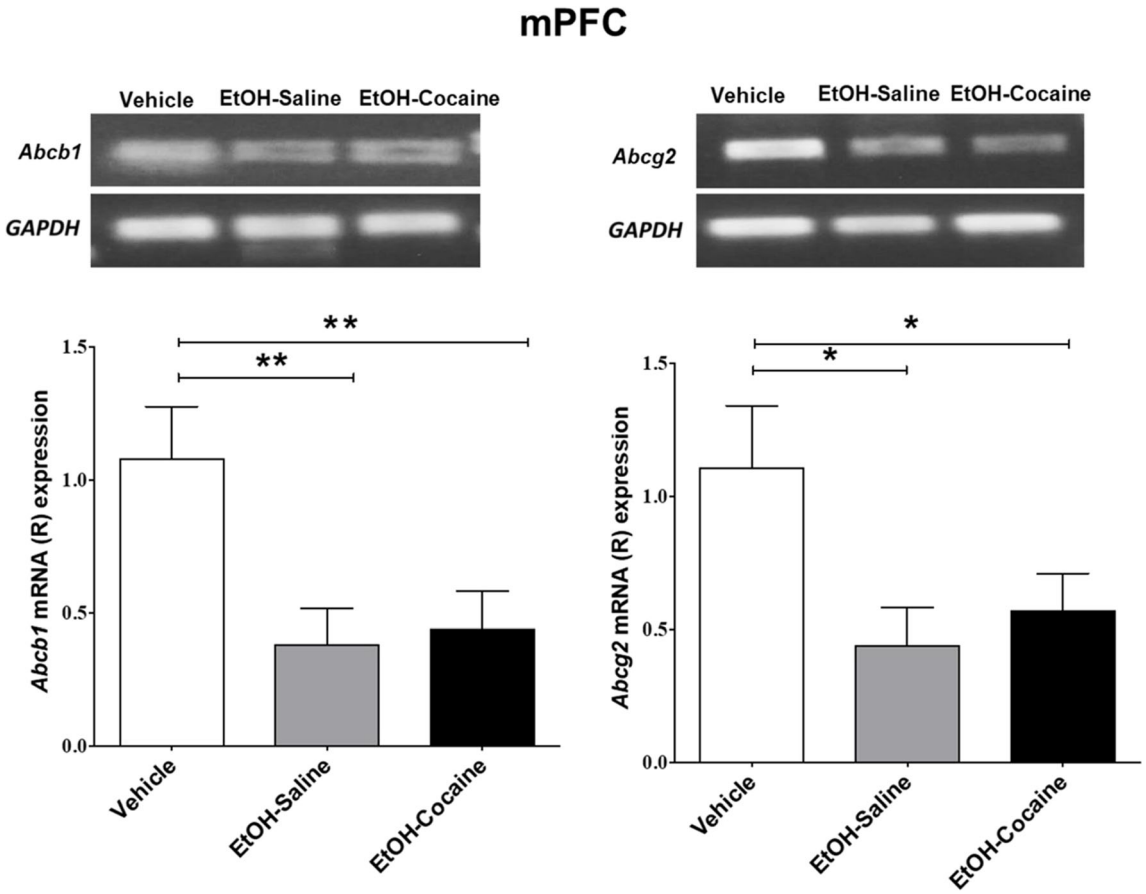

Fig. 3 Relative mRNA expression of *Abcb1* and *Abcg2* in the mPFC following voluntary ethanol consumption and repeated cocaine exposure (mean ± SEM). **a** One-way ANOVA followed by the Newman-Keuls multiple comparisons test revealed a significant decrease in relative mRNA expression of *Abcb1* in the mPFC. **b** One-way ANOVA followed by the Newman-Keuls multiple comparisons test revealed a significant decrease in relative mRNA expression of *Abcg2* in the mPFC. (*$p < 0.05$, **$p < 0.01$), ($n = 5$ for each group)

and ethanol-cocaine treatment alter the expression of mRNA and protein expression of ABCB1 and ABCG2 transporters. The relative mRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ method [44], in which the relative mRNA expression of the desired target is normalized to GAPDH. This was performed to correct for variations in the amount of cDNA added for each sample and to decrease the differences caused by the cycling process and PCR set-up, parallel to a previous published report [45]. Similarly, protein expression for each target was normalized to GAPDH as a loading control widely used for whole cell lysate, and the results were presented as a percentage of the ratio of tested protein/ GAPDH, relative to ethanol-naïve (water) control groups (100% control value) similar to previously published papers [25, 34, 38].

Ethanol-cocaine treatment did not produce any further increases or decreases in *Abcb1*/ABCB1 or *Abcg2*/ABCG2

Table 3 Relative mRNA expression of *Abcb1* and *Abcg2* in the mPFC following voluntary ethanol consumption and repeated cocaine exposure expressed as as mean ± SD.

Abcb1 mRNA expression				Abcg2 mRNA expression			
Vehicle		EtOH-saline	EtOH-cocaine	Vehicle		EtOH-saline	EtOH-cocaine
0.75		0.50	0.66	1.39		0.38	0.27
1.47		0.36	0.49	1.28		0.49	0.62
1.24		0.30	0.63	0.47		0.68	0.82
1.44		0.30	0.34	0.70		0.24	0.91
0.51		0.67	0.30	1.72		0.43	0.24
Mean ± SD	1.08 ± 0.43	0.43 ± 0.16	0.48± 0.16	Mean ± SD	1.11 ± 0.51	0.44 ± 0.16	0.57 ± 0.31

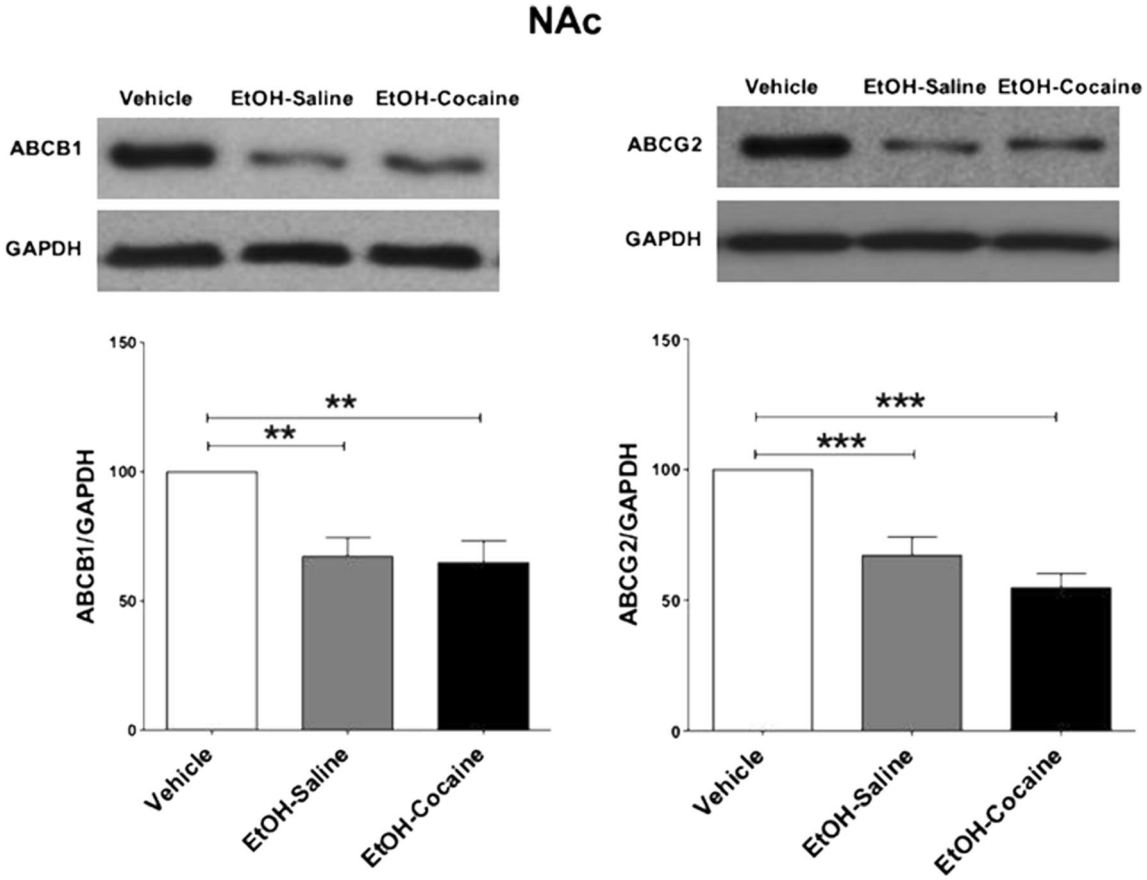

Fig. 4 ABCB1 and ABCG2 protein expression in the NAc following voluntary ethanol consumption and repeated cocaine exposure (mean ± SEM). **a** One-way ANOVA followed by the Newman-Keuls multiple comparisons test revealed a significant decrease in ABCB1 expression in the NAc. **b** One-way ANOVA followed by the Newman-Keuls multiple comparisons test revealed a significant decrease in ABCG2 expression in the NAc. (**$p < 0.01$, ***$p < 0.001$), ($n = 5$ for each group)

expression compared to ethanol treatment alone. Importantly, no statistical significance was shown while comparing the expression of mRNA and protein expression of ABCB1 and ABCG2 transporters in the NAc and the mPFC between ethanol-saline and ethanol-cocaine groups. However, more research is required to investigate the effects of cocaine on these transporters and the timing of changes in mRNA and protein expression relative to different treatment regimens. It remains to be seen whether cocaine treatment alone will produce similar effects to ethanol or whether combinatorial actions might occur depending on the dose and treatment regimen. Moreover, the mechanisms underlying these actions remain to be determined. In this study a three bottle consumption procedure (15 and 30% ethanol concurrently with water) was used for the voluntary home-cage exposure for a period of 5 weeks. The use of two concentrations of ethanol together with water has been proven to increase free ethanol intake [46]. This model of voluntary exposure was used in many

Table 4 Relative protein expression for ABCB1 and ABCG2 in the NAc following voluntary ethanol consumption and repeated cocaine exposure expressed as mean ±SD.

ABCB1/GAPDH				ABCG2/GAPDH			
Vehicle		EtOH-saline	EtOH-cocaine	Vehicle		EtOH-saline	EtOH-cocaine
100		81.97	36.04	100		89.31	44.17
100		61.40	84.28	100		48.55	41.49
100		55.54	63.89	100		56.86	71.11
100		86.97	79.72	100		74.49	53.39
100		50.91	60.02	100		67.14	63.57
Mean ± SD	100±0.00	67.36 ± 16.15	64.79 ± 19.06	Mean ± SD	100±0.00	67.27 ± 15.79	54.75 ± 12.60

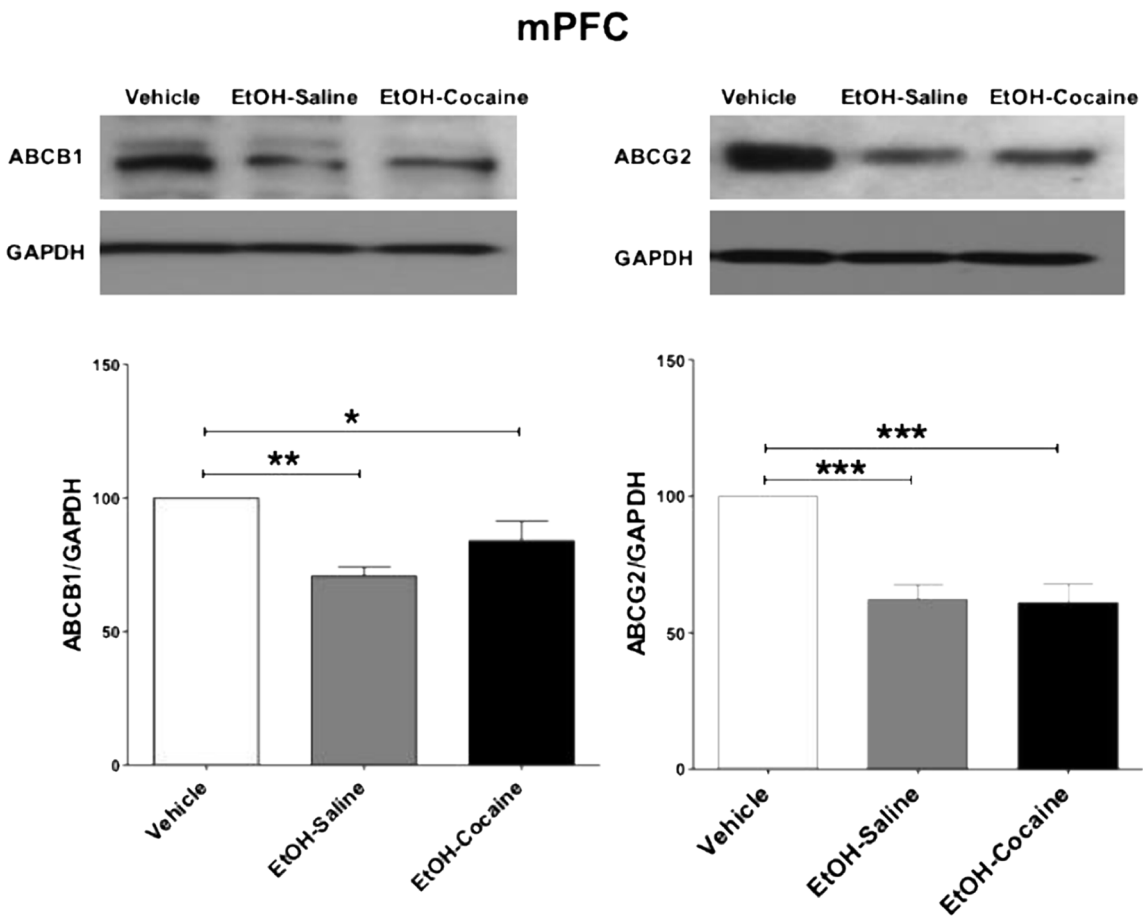

Fig. 5 ABCB1 and ABCG2 protein expression in the mPFC following voluntary ethanol consumption and repeated cocaine exposure (mean ± SEM). **a** One-way ANOVA followed by the Newman-Keuls multiple comparisons test revealed a significant decrease in ABCB1 expression in the mPFC. **b** One-way ANOVA followed by the Newman-Keuls multiple comparisons test revealed a significant decrease in ABCG2 expression in the mPFC. (*$p < 0.05$, **$p < 0.01$,***$p < 0.001$), ($n = 5$ for each group)

studies from our laboratory to study factors that influence ethanol consumption and mitigate excessive ethanol intake in P rats [34, 36, 47]. The cocaine dose for co-exposure was based on studies that have established that repeated cocaine exposure (20 mg/ kg, i.p.) changes the clearance and release of different neurotransmitters in the brain, including glutamate and dopamine [48, 49]. Moreover, the repeated cocaine exposure (20 mg/kg, i.p.) has been shown to downregulate the protein expression of glial glutamate transporters [33].

Previous studies have shown that exposure to different drugs of abuse, including ethanol, cocaine, and nicotine alter neurotransmission and within the neural circuitry underlying drug reward and reinforcement. These changes include altered expression of various proteins regulating neurotransmission in

Table 5 Relative protein expression for ABCB1 and ABCG2 in the mPFC following voluntary ethanol consumption and repeated cocaine exposure expressed as as mean ± SD.

ABCB1/GAPDH				ABCG2/GAPDH			
Vehicle		EtOH-saline	EtOH-cocaine	Vehicle		EtOH-saline	EtOH-cocaine
100		56.87	89.02	100		55.18	54.11
100		72.65	107.85	100		57.00	68.38
100		75.08	67.97	100		76.95	77.68
100		73.98	68.99	100		48.79	38.76
100		75.50	85.92	100		73.18	66.77
Mean ± SD	100±0.00	70.8 2± 7.87	83.95 ± 16.43	Mean ± SD	100±0.00	62.22 ± 12.19	61.14 ± 15.07

both female and male rats [33, 50]. On this basis it would be potentially expected that alterations in ABCB1 and ABCG2 transporters would be observed in both sexes. More studies are warranted in order to verify this effect on female rats. The 75-day-old rats were chosen in order to examine the effect of exposure to drugs of abuse on adults rather than on adolescents. With regard to the issue of age, although similar effects of drugs of abuse were shown on the expression of different transporters, including glial glutamate transporters in adolescents and adults [25, 33, 50], adolescent animals were shown to consume more ethanol than adults [51–53]. Additional research will certainly be needed to determine if the sensitivity of ABC transporters to exposure to drugs of abuse is greater in adolescence, a period in which animals have shown altered sensitivity to drugs of abuse.

A number of studies have shown that transcriptional factors regulate the expression of ABC transporters, including ABCB1 and ABCG2, notably the aryl hydrocarbon receptor (AHR), the pregnane xenobiotic receptor (PXR), and the constitutive and rostane receptor (CAR) [54, 55]. Furthermore, *AHR* mRNA has been detected in the BBB of humans, whereas *PXR* and *CAR* have not been detected in the microvessels of the brain [56]. Nonetheless, CAR and PXR have been found to play a critical role in regulating the expression of ABCB1 and ABCG2 in the BBB [57, 58]. It has been reported that chronic exposure to ethanol (50 mM for 7 days + 200 mM for 6 h) significantly reduces the expression and availability of AHR in mice [19]. Furthermore, exposure to ethanol (200 mM) for 4 h significantly reduces the binding of AHR [59]. These results suggest that chronic exposure to ethanol reduces the availability and the activity of AHR, which in part may decrease the gene and protein expression of *Abcb1*/ABCB1 or *Abcg2*/ABCG2. The exposure of rats to ethanol (4 g/kg, p.o.) for 5 weeks significantly decreased the relative mRNA expression of PXR [60], suggesting that persistent daily ethanol exposure induces a reduction in the expression of this regulator of ABC transporters. More directly, it has been reported that the incubation of cortical progenitor cells with ethanol (120 and 620 mg/dL) significantly reduces the cellular expression of ABCG2 in vitro [16].

It is also possible that the ethanol-induced decrease in the expression of ABCB1 and ABCG2 transporters could result from ethanol-induced neuroinflammation [20, 21]. For example, ethanol exposure significantly increases the levels of pro-inflammatory cytokines, which are known to alter the expression of ABCB1 and ABCG2 [21]. In addition, chronic exposure (5 g/kg/day, i.g. for 10 days) may also induce neuroinflammation by stimulating the production of tumor necrosis factor-alpha (TNF-α) in the brain [61, 62]. Indeed, increased brain TNF-α has been found to be correlated with a decrease in ABCB1 and ABCG2 expression [21, 63]. Interleukin-1β (IL-1β) levels and mRNA expression in the brain were increased in animals exposed to ethanol compared to ethanol-naïve animals [62, 64, 65]. IL-1β could also reduce the expression of ABCB1 and ABCG2, as well as other ABC proteins [21, 66]. In the present study, we found that the ethanol downregulated the relative mRNA and protein expression of *Abcb1*/ABCB1 and *Abcg2*/ABCG2 in both the NAc and mPFC. This effect may be mediated, in part, by an increase in the levels of neuroinflammatory cytokines in the brain, although this remains to be examined.

The expression of ABC transporters may also be regulated more broadly by the immune system. There is a significant positive correlation between chemokine receptor-4 (CXCR-4) overexpression and ABCB1 overexpression in non-small cell carcinoma [31] and peripheral blood mononuclear cells [67]. Inhibition of the CXCR-4 receptor decreases resistance to doxorubicin, which is an ABCB1 substrate [31]. Cocaine modulates the immune system, in part, by suppressing $CD4^+$ T cell function [68]. Furthermore, in vitro, cocaine exposure at a dose of 10^{-9} to 10^{-4} M inhibits the migration of human fetal brain-derived neural precursor cells in response to the chemokine CXCL-12 and exposure at a concentration of 10^{-6} M for 7 days downregulates CXCR-4 [29]. Importantly, CXCR-4 induces the expression of c-Jun and consequently upregulates the expression of ABCG2 [30]. Thus, it is possible that the effects of cocaine on ABCB1 and ABCG2 transporters in our study could result from alterations in chemokines, although this remains to be determined.

Modulation of ABC transporters by drugs of abuse may have wide-ranging implications for the treatment of diverse conditions with drugs that are substrates of these transporters, as well as for addiction. A history of illicit drug use might influence responses to other medications by altering tissue penetration and other pharmacokinetic properties of drugs that are substrates of these transporters. The present data suggest that there are alterations in brain expression of these transporters, but it remains highly probably that there are alterations in the expression of these transporters in other tissues involved in the excretion of these drugs, including cancerous tissues for which the expression of these transporters is an important aspect of drug resistance. The converse may also be true that exposure to other drugs that influence the expression of these transporters might subsequently influence responses to drugs of abuse. It remains to be determined if the changes in the expression of these transporters are associated with changes in pharmacokinetic properties of drugs of abuse, and in particular brain penetration, and whether such changes might influence the subsequent behavioral and psychological impact of drugs as part of the addictive process.

The idea that the level of expression of these transporters might play a role in drug dependence is supported by other findings. The ABC transporter *ABCC4* has been repeatedly found to be associated with drug dependence in genome-wide association studies (GWAS) [69]. Particular transporters may also be more specifically related to dependence to

particular drugs or drug classes. *ABCB1* markers have been repeatedly associated with opiate dependence [70]. There is also some suggestion that it may be associated with ethanol dependence [71]. Although the association did not reach "genome-wide" significance in that study, other approaches in animals also identified the homologous gene as being associated with responses to ethanol [72, 73]. A meta-analysis of nicotine dependence studies, seeking to identify pathways involved in the liability to nicotine dependence, identified a cluster of genes involved in xenobiotic signaling, including *ABCB1*, *AHR*, and *TNF* [74]. As it is the case for many genes associated with drug dependence, the genetic relationship may not be direct, but might be associated with another endophenotype or psychiatric co-morbidity, such as antisocial behavior, which has been associated with *ABCB1* markers [75]. Most importantly, the deletion of *Abcb1* or *Abcg2* in mice has been shown to increase both blood and brain levels of Δ-9-tetrahydrocannibinal (THC) and to potentiate the THC-induced hypothermia [76], and deletion of *Abcb1* potentiates the respiratory depressive effects of buprenorphine by reducing brain efflux of norbuprenorphine [77].

In summary, our work sheds the light on the direct effect of voluntary ethanol consumption (with or without repeated cocaine exposure) on the expression of ABC efflux transporters in central brain regions involved in drug reward and reinforcement. Our work provides information about some possible drug-drug interactions among the substrates of the ABC transporters, including drugs of abuse in the brain. However, this area of research is not well-studied and needs further investigation, as it has important potential implications for mechanisms that may contribute to the addictive process, as well as for the use of drugs in individuals with a history of drug abuse. Future studies are warranted to investigate the effects of chronic exposure of ethanol, cocaine, and other abused drugs on the transcriptional factors that regulate ABC transporters and other potential mediators of these effects, such as neuroinflammation and immune factors. Moreover, additional research is required to determine the relationship between ABC transporter expression and function, and behavioral and psychological responses to drugs of abuse.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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